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Kirkegaard, Signe Skyum; Strøm, Pernille Dyhl; Gammeltoft, Steen; Hansen, Anker Jon; Hoffmann, Else Kay

*Published in:*  
Cellular Physiology and Biochemistry

*DOI:*  
[10.1159/000443042](https://doi.org/10.1159/000443042)

*Publication date:*  
2016

*Document version*  
Publisher's PDF, also known as Version of record

*Document license:*  
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*Citation for published version (APA):*  
Kirkegaard, S. S., Strøm, P. D., Gammeltoft, S., Hansen, A. J., & Hoffmann, E. K. (2016). The volume activated potassium channel KCNK5 is up-regulated in activated human T Cells, but volume regulation is impaired. *Cellular Physiology and Biochemistry*, 38(3), 883-892. <https://doi.org/10.1159/000443042>

Original Paper

# The Volume Activated Potassium Channel KCNK5 is Up-Regulated in Activated Human T Cells, but Volume Regulation is Impaired

Signe S. Kirkegaard<sup>a</sup> Pernille Dyhl Strøm<sup>a</sup> Steen Gammeltoft<sup>b</sup> Anker Jon Hansen<sup>c</sup>  
Else K. Hoffmann<sup>a</sup>

<sup>a</sup>Section for Cell and Developmental Biology, University of Copenhagen, Copenhagen, <sup>b</sup>Department of Clinical Biochemistry, Glostrup Hospital, Glostrup, <sup>c</sup>Novo Nordisk, Måløv, Denmark

## Key Words

KCNK5 • T cell activation • CD3/CD28 • T cells • RVD

## Abstract

**Background/Aims:** The potential role of the two-pore domain potassium channel KCNK5 (also known as TASK-2 and  $K_{2p}5.1$ ) in activated T cell physiology has only recently been described. So far KCNK5 has been described to be up-regulated in T cells in multiple sclerosis patients and to be implicated in the volume regulatory mechanism regulatory volume decrease (RVD) in T cells. **Methods:** We investigated the time-dependent expression pattern of KCNK5 in CD3/CD28 activated human T cells using qPCR and Western blotting and its role in RVD using a Coulter Counter. **Results:** KCNK5 is highly up-regulated in CD3/CD28 activated T cells both at mRNA (after 24 h) and protein level (72 and 144 h), but despite this up-regulation the RVD response is inhibited. Furthermore, the swelling-activated  $Cl^-$  permeability in activated T cells is strongly decreased, and the RVD inhibition is predominantly due to the decreased  $Cl^-$  permeability. **Conclusion:** The up-regulated KCNK5 in activated human T cells does not play a volume regulatory role, due to decreased  $Cl^-$  permeability. We speculate that the KCNK5 up-regulation might play a role in hyperpolarization of the cell membrane leading to increased  $Ca^{2+}$  influx and proliferation of T cells.

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## Introduction

Ion channels are important for T cell activation and activated T cell function. Especially the role of the voltage-gated  $Kv1.3$ , the  $Ca^{2+}$ -activated  $KCa3.1$  and  $Ca^{2+}$  release-activated  $Ca^{2+}$  (CRAC) channel is well known. For reviews on ion channels and T cells see [1-3]. Activation of T cells when encountering an antigen requires a sustained increase in intracellular  $Ca^{2+}$

Else K. Hoffmann, PhD

Section for Cell and Developmental Biology, Department of Biology, The August Krogh Building, University of Copenhagen, 13, Universitetsparken, DK-2100, Copenhagen, (Denmark); E-Mail ekhoffmann@bio.ku.dk

concentration ( $[Ca^{2+}]_i$ ) which is generated by  $IP_3$  mediated  $Ca^{2+}$  release from stores in the ER which in turn mediates the activation of CRAC channels allowing  $Ca^{2+}$  entry from the extracellular medium. This  $Ca^{2+}$  influx and subsequent rise in  $[Ca^{2+}]_i$  activates KCa1.3 and also causes a depolarization activating the voltage sensitive Kv1.3  $K^+$  channel. Activation of Kv1.3 and KCa3.1 causes  $K^+$  efflux and hyperpolarization of the plasma membrane allowing further  $Ca^{2+}$  entry via the CRAC channels in the membrane.

The sustained increase in  $[Ca^{2+}]_i$  is essential for the transcription and expression of interleukin 2 (IL-2) which in turn is vital in keeping the T cells activated without the requirement of further antigen stimulation (see [4]). Other ion channels, however, have been implicated in T cell activation. The leak conductance channel (TREK-2) was described in WEHI-231 cells which is a murine immature B cell line [5, 6]. Further studies have also revealed the presence of two-pore domain  $K^+$  channels in lymphocytes e.g. TASK-1, 2 (KCNK5) and 3 [7]. Of special interest to this study Bittner and co-workers suggested that the two-pore domain potassium channel KCNK5 (also known as TASK-2 or  $K_{2p}5.1$ ) has a role in T cell activation and in multiple sclerosis [8].

T cells are in constant movement around the body and thereby subjected to extracellular osmotic changes e.g. in the kidney. T cells are, as most other mammalian cells, capable of performing RVD (regulatory volume decrease) in response to cell swelling [9-12].

KCNK5 has previously been shown to be volume sensitive and to be implicated in the volume regulatory process of RVD in response to cell swelling in various cell types and tissues including Ehrlich Ascites Tumor (EAT) cells [13, 14], mouse proximal tubules [15], human and murine spermatozoa [16, 17] and murine [18] and human T lymphocytes [1]. For a recent review on KCNK5 see [19]. Other types of  $K^+$  channels have been suggested to play a part in T cell volume regulation including Kv1.3 [20, 21] and KCa3.1 [22]. However, volume regulation also requires the transport of anions, which includes the volume regulated anion channel (VRAC) responsible for the swelling-activated  $Cl^-$  current [2].

KCNK5 is up-regulated during T cell activation [8, 23] but there is, to our knowledge, no description of the time dependency of the expression pattern of KCNK5 during T cell activation and its physiological consequences. This is the subject of the present paper, and in addition we demonstrate a strong decrease in the swelling-activated  $Cl^-$  permeability during the activation, which inhibits the RVD process in the large activated T cells.

## Materials and Methods

### *Solution and Materials*

**Volume measurements:** Hypotonic Ringer's solution (160 mOsm) contained (in mM): 71.5 NaCl, 2.5 KCl, 0.5  $MgSO_4$ , 0.5  $Na_2HPO_4$ , 0.5  $CaCl_2$ , 3.3 MOPS, 3.3 TES and 5 HEPES, pH 7.4. Isotonic Ringer's solution (300 mOsm) was obtained by addition of sucrose.  **$Cl^-$  permeability:** the hypotonic (150 mOsm) low  $Na^+$  Ringer's solution contained (in mM): 70 NMDG (N-methyl-D-glucamine)  $Cl^-$ , 0.8 NaCl, 5 KCl, 1  $K_2HPO_4$ , 1  $MgSO_4$ , 1  $CaCl_2$ , 3.3 MOPS, 3.3 TES and 5 HEPES, pH 7.4.

Gramicidin and Clofilium was purchased from Sigma and used in the concentrations 1  $\mu M$  and 100  $\mu M$  respectively

### *Purification and maintenance of cells*

Human T cells were purified from buffy coats from healthy donors. Human buffy coats were obtained from the blood bank at Rigshospitalet, Copenhagen, Denmark. All procedures were performed at room temperature. The T cell population, CD4 and CD8 subtypes were purified from buffy coats using RosetteSep™ (Human T Cell Enrichment Cocktail, Human CD4+ T Cell Enrichment Cocktail and Human CD8 T Cell Enrichment Cocktail all from StemCell Tech.) according to manufactures description. After purification any residual red blood cells was lysed using RBC lysis buffer (eBioscience). Purified T cells, CD4 or CD8 subtypes were kept in RPMI<sub>1640</sub> GlutaMax medium supplemented with 30 U/ml IL-2, 10% FBS and 1% P/S at 37°C and 5%  $CO_2$ .

## RNA purification and qPCR

T cells were stimulated for 2, 4, 8, 12, 24, 48, 72 and 144 hours with CD3/CD28 beads (Invitrogen) in the ratio 1:1 (according to manufacturer's instruction) and RNA was purified from lysates using NucleoSpin® RNA II (Macherey-Nagel) according to manufacturer's instructions. Reverse transcriptase PCR with SuperScript II (Invitrogen) and oligo(dT)<sub>12-18</sub> primer (Invitrogen) was used to generate cDNA from the purified mRNA and performed on a Eppendorf Mastercycler as previously described [24]. QPCR was performed using a Stratagene MX4000 real-time PCR system, Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies) and the following primers: hKCNK5 forward: 5'-ACCACCCACTCATCTTCCAG-3', hKCNK5 reverse: 5'-AGTGCTGGTGAAGGTGGACT-3', hKv1.3 forward: 5'-CACTTCAGGTTTCAGCAGCA-3', hKv1.3 reverse: 5'-TGCTCCCGGTGGTAGAAGT-3', hKCa3.1 forward: 5'-ACTGGGCACCTTTCAGACAC-3' and KCa3.1 reverse: 5'-ACGTGCTTCTCTGCCTTGT-3'. A total volume of 20 µl containing 1 µl of the cDNA, 200 nM of primers, and 10 µl 2× MasterMix was used.

Despite testing numerous potential reference genes none of them lived up to our standards and instead determination of total cDNA concentration was used to correct the data obtained. To determine cDNA content upon PCR reaction and to normalize samples, Quant-iT™ OliGreen® ssDNA Assay Kit was used as described in [25]. Standard curves were also done to measure primer efficiency which was corrected for in calculations. Primers were selected using Primer3 software and purchased from MWG Eurofins (Germany). Quantification was carried out using the Pfaffl method [26].

## Western blotting

T cells were stimulated for 2h, 4h, 8h, 12h, 24h, 48h, 72h or 144h days with CD3/CD28 beads and lysed in 95°C lysis buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 20 mM EDTA) with protease inhibitors (Roche Applied Science) and phosphatase inhibitors added. SDS-PAGE and Western blotting was performed as previously described [13], where protein concentration measurements assured that an equal amount of protein was loaded into every well. We used the following antibodies and concentrations: KCNK5 (TASK-2) (Alomone Lab., Israel) 1:100, β-actin (Sigma-Aldrich) 1:1000.

## Volume measurements by Coulter Counter

**Volume measurements:** T cells were stimulated with CD3/CD28 beads for 2, 4, 8, 12, 24, 48, 72 or 144 hours before the absolute cell volume was measured by electronic cell sizing using the Coulter Multisizer II (Coulter, Luton, UK) with a tube orifice of 70 µm. Before measurements calibration was performed using 15 µm latex beads. Approximately  $2.5 \times 10^6$  cells were used per experiment and cell volume was determined in either isotonic or hypotonic Ringer's solutions (all micro-filtered before use). Volume recovery was estimated in two different ways, by % recovery after 3 min. as previously described [13] and by calculating the initial rate of RVD measured as the slope of the recovery part of the RVD curve.

**Cl<sup>-</sup> permeability:** to determine if the Cl<sup>-</sup> permeability was decreased in activated T cells we used a method previously described in [27] with some minor alterations. Cell volume regulation (i.e. RVD) requires efflux of both cations and anions. We employed Clofilium as a blocker of potassium channel KCNK5 and substituted most of sodium with NMDG in the Ringer's solution (see above), this was done in order to obtain equilibrium for Na<sup>+</sup>, thus ensuring no net movement. The principle of the experiments is as follows: Clofilium blocks the KCNK5 channel and hence RVD. When Gramicidin is added high cation permeability is introduced making Cl<sup>-</sup> the limiting factor. The procedure makes it possible to assess the relative Cl<sup>-</sup> permeability.

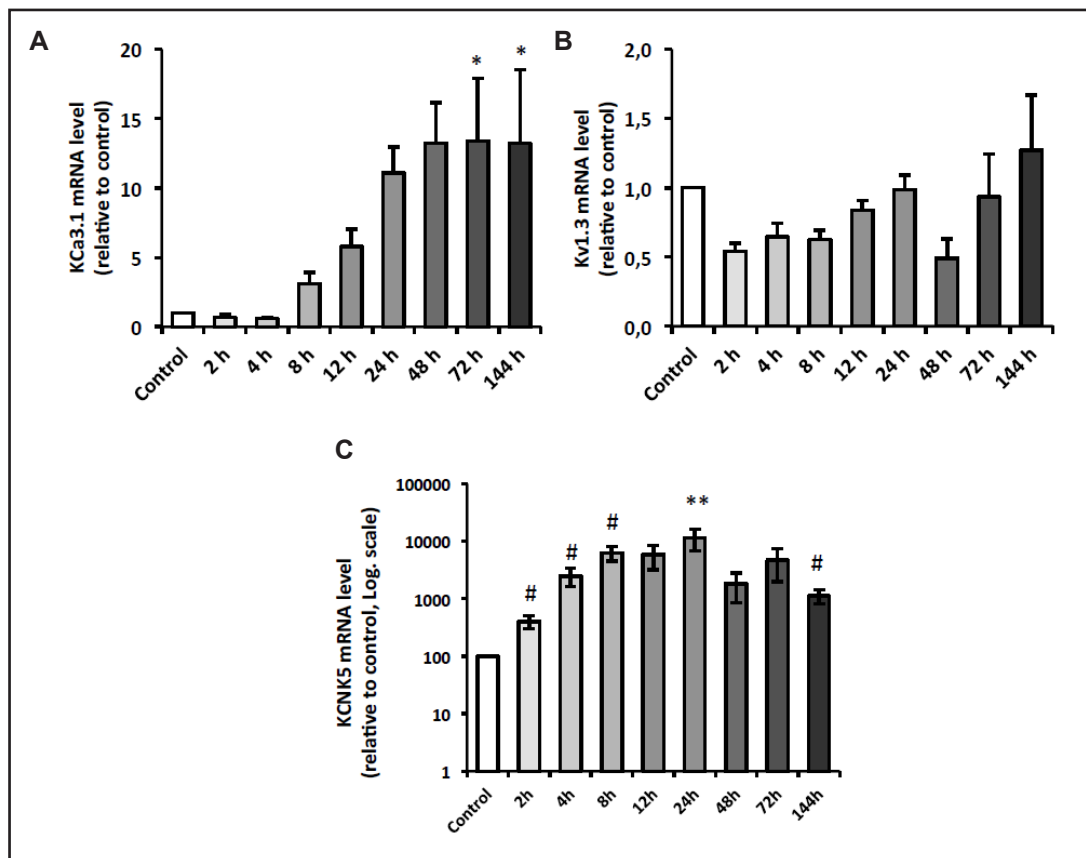
## Statistical methods

Statistical significance was determined by Student's T-test or one-way analysis of variance (ANOVA) where one star represent statistical significance at a 95% level, two stars at a 99% level and three stars at a > 99% significance level.

## Results

### KCNK5 and K<sub>Ca</sub>3.1 mRNA's are up-regulated in activated T cells

Activation of T cells for 2, 4, 8, 12, 24, 48, 72 or 144 hours with CD3/CD28 beads caused up-regulation of KCa3.1 mRNA (Fig. 1A) after 72h and 144h (ANOVA) as well as KCNK5

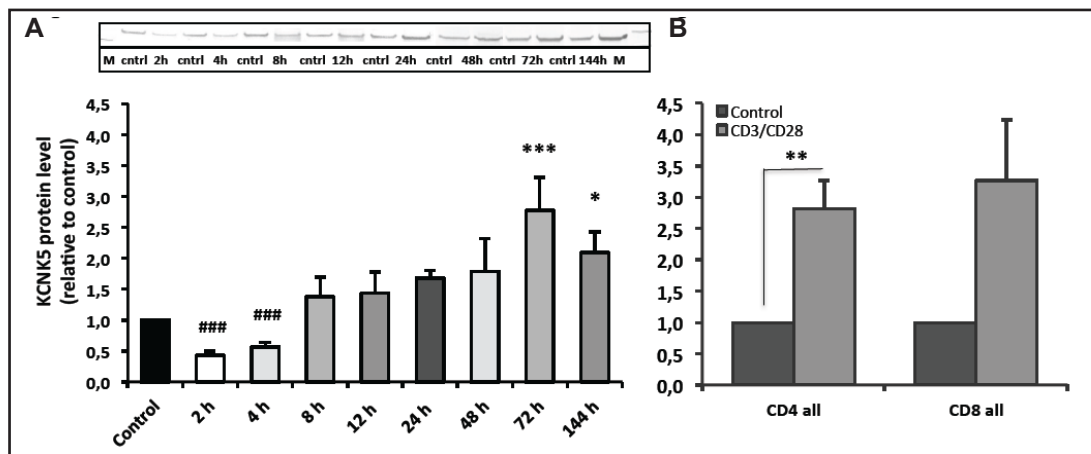


**Fig. 1.** mRNA levels of KCNK5, KCa3.1 and Kv1.3 determined by qPCR. T cells were purified from human buffy coats and stimulated with CD3/CD28 activation beads for 2h, 4h, 8h, 12h, 24h, 48h, 72h or 144h. mRNA levels were determined using qPCR and primers against KCa3.1 (A), Kv1.3 (B) and KCNK5 (C). ANOVA (\*) and student's t-test (#) was used to test for statistical significance on 5-8 independent experiments (n = 2 for 48h). One star (\*) or # indicates a 95% significance level and two stars indicate significance at a 99% level.

mRNA after 2 (student's t-test), 4 (student's t-test), 8 (student's t-test), 24 (ANOVA) and 144 h (student's t-test) (Fig. 1C) while no significant change was seen in regard to Kv1.3 (Fig. 1B). A tendency to initial down-regulation of KCa3.1 (Fig. 1A) mRNA level was observed upon 2 and 4 h of stimulation followed by a tendency to increasing up-regulation after 8, 12 and 24 h reaching a significant level of a 13 fold up-regulation after 72 h. KCNK5 (Fig. 1C) showed the largest up-regulation of the three potassium channels studied with a 115 fold peak-increase in mRNA level after 24 h of activation. There was no initial down-regulation thus a significant (T-test) 4 fold increase was already seen after 2 h of CD3/CD28 stimulation.

#### *KCNK5 protein is up-regulated in activated T cells*

When measuring KCNK5 protein levels a biphasic protein expression pattern during activation with CD3/CD28 beads (Fig. 2A) was seen. There was an initial significant decrease ( $p < 0.001$ , student's t-test) at 2 and 4h followed by a tendency to increasing protein expression after 8, 12, 24 and 48 h (though not statistically significant) peaking at 72 h with a significant 277% (ANOVA) increase compared to non-stimulated cells. A subsequent slight decrease of KCNK5 protein expression to 209 % at 144 h was furthermore seen compared to the quiescent control cells. The activation of T cells stimulated proliferation causing a more than three-fold increase upon 72 h of CD3/CD28 stimulation compared to non-stimulated control cells (two experiments, data not shown). When comparing the up-regulation of the



**Fig. 2.** KCNK5 protein expression. T cells whole population (Fig. 2A) and CD4 and CD (subtypes) (Fig. 2B) were purified from human buffy coats and stimulated with CD3/CD28 activation beads (CD4 and CD8 subtypes were only stimulated for 144 h) for 2h, 4h, 8h, 12h, 24h, 48h, 72h or 144h. SDS-PAGE and Western blotting was performed using antibodies against KCNK5 and  $\beta$ -actin. Protein bands were visualized and pixel density calculated. ANOVA (\*) or student's t-test (#) was used to determine statistical significance on 3-10 independent experiments and one star (\*) represents a statistical significance level of 95% whereas (\*\*\*) indicate a significance level > 99%. (Fig. 2A) No significance was seen after 2 and 4 h using the statistical test ANOVA but when using Student's T-test both times showed significance here represented by (###) indicating a level of significance of >99%. (Fig. 2B) Stimulation with CD3/CD28 beads for 3 days increased KCNK5 protein expression in both CD4 and CD8 subtypes, but no significant difference in KCNK5 expression was observed between the two T cell subtypes.

two T-cell subtypes CD4 and CD8 (stimulated for 144 h) no significant difference in KCNK5 protein expression level was observed (Fig. 2B).

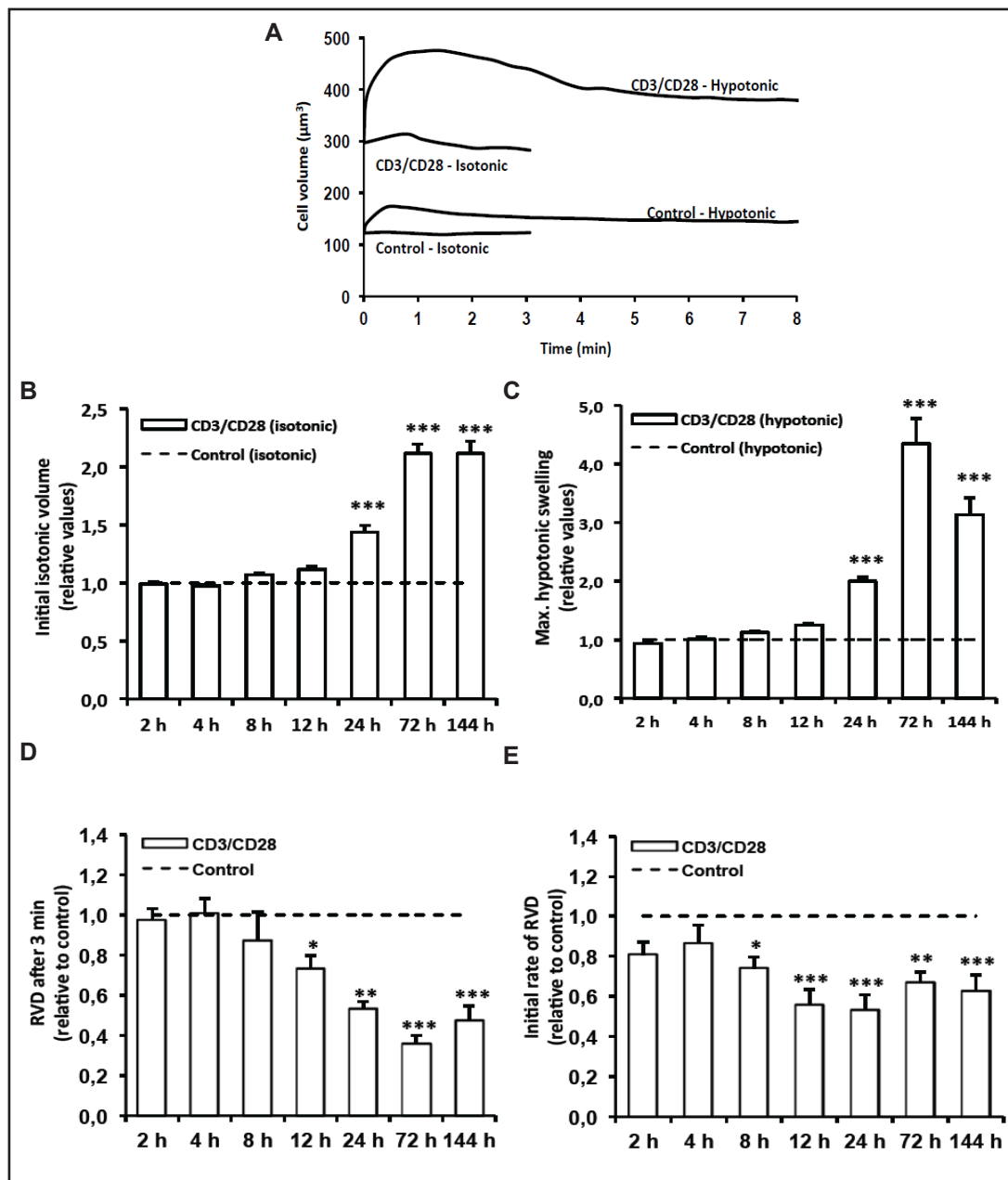
*T cell activation causes increased cell volume, decreased RVD ability and increased hypotonic swelling*

Since the KCNK5 channel is a known volume regulator we tested the ability of activated T cells to perform regulatory volume decrease (RVD). The volume measurements depicted in Fig. 3 (relative values) and table 1 (absolute values) show the well-known fact that activated T cells become larger (Fig. 3A+B and Table 1). After 8 h of CD3/CD28 stimulation T cells in isotonic Ringer's solution began increasing their volume and after 24 h the mean volume was significantly ( $p < 0.001$ ) larger than the control cells with a mean volume of  $172 \mu\text{m}^3$  compared to  $119 \mu\text{m}^3$  of the control cells (Table 1). There was a subsequent increase of cell volume to more than 200 % at 72 and 144 h (Fig. 3B).

Figure 3A shows the time course of the cell volume of activated (144 h) and non-activated cells when exposed to a hypotonic Ringer's solution. CD3/CD28 stimulated T cells had increased maximum swelling which was significant after 24 h, with highest measured values after 72 h and a small decrease seen after 144 h. At 72 h their maximum swelling reached a value of  $309 \mu\text{m}^3$  compared to a gain of volume of  $72 \mu\text{m}^3$  seen in control cells equivalent to a 4 times increase of cell volume (Fig. 3C and Table 1). Maximum swelling values upon 24, 72 and 144 h of stimulation was significantly larger than control values ( $p < 0.001$ ) both in absolute values and in relative terms (Fig. 3C and Table 1).

Besides a more pronounced swelling when hypotonically challenged, activated T cells showed an impaired RVD performance compared to control cells. Fig. 3A shows that both activated and non-activated T cells swelled and performed RVD. However, % recovery after 3 min. [13] and the initial rate of RVD was much less in stimulated T cells than in control cells (Fig. 3D+E respectively). The RVD response or the recovery percentages of non-activated T cells was not significantly different from each other at the measured time-points (average recovery of 36 % after 3 min). Fig. 3D and Table 1 illustrates that the RVD of activated T cells



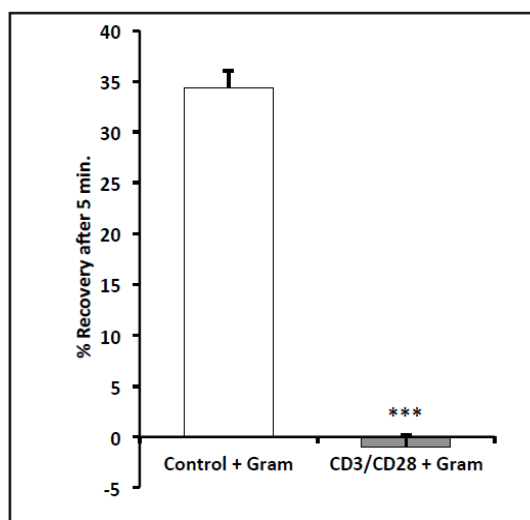


**Fig. 3.** RVD in CD3/CD28 stimulated T cells and non-activated controls. T cells were purified from human buffy coats and stimulated with CD3/CD28 activation beads for 2, 4, 8, 12, 24, 72 or 144 hours before absolute volume was measured using a coulter counter. Stimulated T cells and non-stimulated controls were measured in either isotonic (300 mOsm) or hypotonic (160 mOsm) Ringer's solution. (A) representative figure showing RVD over time (min) for untreated control cells and CD3/CD28 stimulated T cells (144 hours). (B) Initial mean volume measured in isotonic Ringer's solution (C) Mean maximal swelling seen in hypotonic Ringer's solution determined as  $V_{\max} - V_{\text{iso}}$  where  $V_{\max}$  is the maximal swelling seen when subjecting the cells to hypotonicity and  $V_{\text{iso}}$  is the volume seen in isotonic Ringer's solution. (D) Mean volume recovery after 3 min was calculated as  $(V_{\max} - V_{3\text{min}}) / (V_{\max} - V_{\text{iso}})$ , where  $V_{\max}$ ,  $V_{3\text{min}}$  and  $V_{\text{iso}}$  are the maximal cell volume, cell volume at time 3 min and cell volume under isotonic conditions, respectively. (E) initial rate of RVD (the slope) was calculated using linear regression on the linear part of the RVD curve, from maximum volume to the end of linearity. ANOVA test was used to test for statistical significance on 5-7 independent experiments and one star (\*) represent a 95% significance level whereas two (\*\*) and three stars (\*\*\*) indicate significance levels of 99% and > 99% respectively.

**Table 1.** Volume measurements in absolute values. Mean values of isotonic initial volume, max. swelling upon hypotonicity, % recovery and initial rate of RVD  $\pm$  SD showed in absolute values (Fig. 3 shows the same results in relative values)

	Volume measurements in absolute values							
	Initial volume (iso) ( $\mu\text{m}^3$ )		Max. swelling (hypo) ( $\mu\text{m}^3$ )		RVD (%)		Initial rate of RVD $\mu\text{m}^3/\text{sec}$	
	Control	CD3/CD28	Control	CD3/CD28	Control	CD3/CD28	Control	CD3/CD28
2h	122.7 $\pm$ 8.69	122.6 $\pm$ 10.5	68 $\pm$ 8.69	60.9 $\pm$ 13.5	38.96 $\pm$ 6.09	37.93 $\pm$ 8.11	-0.0025 $\pm$ 5.7e $^{-4}$	-0.002 $\pm$ 6e $^{-4}$
4h	117.5 $\pm$ 9.51	114.8 $\pm$ 11.3	69.3 $\pm$ 11.5	69.4 $\pm$ 9.66	33.68 $\pm$ 7.12	34.38 $\pm$ 11.44	-0.0024 $\pm$ 5e $^{-4}$	-0.0021 $\pm$ 6.9e $^{-4}$
8h	115.1 $\pm$ 9.61	123.1 $\pm$ 11.5	72.7 $\pm$ 10.3	81.1 $\pm$ 7.57	33.92 $\pm$ 6.88	28.35 $\pm$ 10.62	-0.0025 $\pm$ 4e $^{-4}$	-0.0018 $\pm$ 1.8e $^{-4}$
12h	114.1 $\pm$ 9.44	127.5 $\pm$ 14.3	71.7 $\pm$ 12.1	89.2 $\pm$ 12	36.27 $\pm$ 6.47	26.5 $\pm$ 7	-0.0028 $\pm$ 6e $^{-4}$	-0.0015 $\pm$ 3.5e $^{-4}$
24h	118.8 $\pm$ 10.9	171.6 $\pm$ 29.6	63.9 $\pm$ 11.6	127.5 $\pm$ 24	40.68 $\pm$ 5.07	21.85 $\pm$ 5.64	-0.0028 $\pm$ 4.3e $^{-4}$	-0.0015 $\pm$ 4.7e $^{-4}$
72h	110.8 $\pm$ 7.32	237.9 $\pm$ 15.5	71.9 $\pm$ 15.1	309.3 $\pm$ 34	35.7 $\pm$ 5.67	13.61 $\pm$ 4.82	-0.0027 $\pm$ 3.9e $^{-4}$	-0.0019 $\pm$ 3.4e $^{-4}$
144h	112.4 $\pm$ 6.47	237.6 $\pm$ 34.1	69.2 $\pm$ 13.8	209.4 $\pm$ 28.7	32.99 $\pm$ 6.6	15.52 $\pm$ 5.82	-0.0025 $\pm$ 4.1e $^{-4}$	-0.0016 $\pm$ 6e $^{-4}$

**Fig. 4.**  $\text{Cl}^-$  permeability in RVD in CD3/CD28 activated and non-activated T cells. T cells were purified from human buffy coats and stimulated with CD3/CD28 beads for 72 hours. Activated T cells and non-activated control cells were treated with 100  $\mu\text{M}$  Clofilium for 30 min to block the potassium channels. The cells were swollen in hypotonic low  $\text{Na}^+$  NMDG Ringer's solution and their volume was measured using a Coulter Counter. After one min. 1  $\mu\text{M}$  Gramicidin was added to facilitate cation flux thereby letting the  $\text{Cl}^-$  flux being the limiting factor. The figure shows % recovery after 5 min of activated and non-activated T cells treated with Clofilium and Gramicidin. Student's T-test was used to test for statistical significance on 3 independent experiments and two stars (\*\*) indicate a significance level of 99%.



started to decrease after 8 h followed by significant decreases at 12, 24, 72 and 144 h of stimulation. After 72 h of CD3/CD28 stimulation the cells had the lowest ability to perform RVD with a recovery of 13.6 % compared to 35.7 % (Table 1) of the non-stimulated cells and equivalent to a 60 % decrease (Fig. 3D). When measuring RVD by the initial rate of shrinkage the results was similar – activated T cells have a lesser RVD performance than control cells (Fig. 3E and Table 1). The initial rate of RVD was faster in all controls when compared to the activated T cells though the values was only significantly lower after 8, 12, 24, 72 and 144 h.

#### *$\text{Cl}^-$ permeability of activated T cells is inhibited*

The considerable up-regulation of KCNK5 in activated T cells does not increase their ability to perform RVD. On the contrary RVD is diminished in CD3/CD28 activated cells. Since RVD is driven by the extrusion of KCl we speculated if the  $\text{Cl}^-$  permeability of the activated cells could be decreased. As described in materials and methods and in [27] we used the rate of RVD after addition of Gramicidin in a low  $\text{Na}^+$  medium with Clofilium to block the  $\text{K}^+$  channel as a measure of the swelling-activated  $\text{Cl}^-$  permeability. As seen in Fig. 4 the addition of Gramicidin to hypotonically swollen non-stimulated control cells resulted in a significant faster RVD (higher  $\text{Cl}^-$  permeability) with a 34.3  $\pm$  1.02% recovery compared to -0.102  $\pm$  1.24% (statistically not different from zero) seen in the CD3/CD28 activated T cells. Thus there was a significant decrease ( $p < 0.05$ ) in the  $\text{Cl}^-$  permeability in stimulated T cells when comparing them to their non-activated counterparts.



## Discussion

In the current study we find that the activated human T cells enlarge upon activation which is a well described feature in the activated and proliferating T cells [28, 29]. There is a strong up-regulation of KCNK5 which is in agreement with earlier findings [8, 23]. The time profile described here shows an initial (after 2 and 4 hours of CD3/CD28 stimulation) and significant decrease in KCNK5 protein expression in activated T cells which correlates with a minimal not significant early shrinkage and decreased maximum swelling when comparing the activated T cells to non-activated control cells.

CD3/CD28 activated T cells not only get larger when measured in isotonic Ringer's solution they also swell significantly more when subjected to a hypotonic Ringer' solution as if they have an inhibited KCl efflux. Despite the up-regulation of the potent volume regulator KCNK5 we find the activated T cells to have an inhibited RVD performance upon cell swelling compared to the control cells. Since an up-regulation of KCNK5 protein expression and a decreased RVD might seem contradictory we speculate if the up-regulation occurs in order to facilitate proliferation. Proliferating cells are enlarged to keep daughter cells the same size as the parental cell a feature vital for successful proliferation, thus a strong RVD mechanism would be problematic for the activated T cell whose main objective is to increase in size and number to sufficient fight off a pathogenic intrusion. Cell swelling has been shown to facilitate proliferation in different cells [30-32]. The KCNK5 up-regulation will help insure a hyperpolarization of the cell membrane and thus a sustained  $\text{Ca}^{2+}$  influx vital for the long-term activation of the T cells keeping them activated in an antigen-independent manner [33-37] this would facilitate optimal conditions for the T cell expansion. Andronic et al. find the RVD performance in stimulated and non-stimulated T cells to be the same, an observation contradicting our findings [23], but Andronic et al. only measure the 20 min. volume recovery and from their Fig. 2 we calculate the initial rate of volume recovery to be slower in stimulated cells compared to non-stimulated cell as found in the present study. Furthermore they use video microscopy to measure the volume a method quite different from our experimental set-up in which we measure the absolute cell volume of cells in suspension using a Coulter Counter. With respect to maximum swelling we and Deutch and Lee [38] find the stimulated cells to swell more than non-stimulated control cells an observation contradicting what was found by Andronic et al. [23]. We do not have an explanation for these apparent differences. It should be pointed out that the hypotonic and isotonic solutions used for cell volume measurements in the present study contain equal amount of electrolyte. Therefore there were no changes in the extracellular ion concentration during hypotonic treatment only in osmolarity. This is different from the condition used by Bobak et al. [18] and Andronic et al. [23] where hypotonic solutions also had a decreased ion concentration, the possible importance of this difference has not been tested.

Since the RVD in activated T cells was found to be inhibited despite the strong up-regulation of KCNK5 protein we speculated whether the swelling-mediated  $\text{Cl}^-$  permeability in activated T cells could be inhibited thereby setting a limit for the cells RVD capacity. We tested this and found that the  $\text{Cl}^-$  permeability through the volume-sensitive anion channel VRAC indeed was inhibited in the activated T cells compared to control cells. This means that regardless of the increase in KCNK5 protein quantity the cells will not perform RVD as long as there is a functional down-regulation of VRAC since the KCl efflux during RVD is an electroneutral process. It should be noted that our results are in conflict with those published by Deutsch and Lee in 1988 [38] where it is shown how phytohemagglutinin activated human peripheral blood lymphocytes (PBL) respond to Gramicidin in a set-up similar to ours by decreasing cell volume, and it is thus speculated that  $\text{K}^+$  is the rate limiting factor of RVD in activated T cells. A major difference seems to be that Deutsch and Lee are not adding Gramicidin until 30+ minutes after cell swelling whereas we add it immediately after cell swelling when the channels are maximally stimulated. We have previously shown in Ehrlich cells that the volume activated  $\text{Cl}^-$  channels close before 15 min. after swelling activation [27] and Sarkadi and co-workers show that it is also true in human lymphocytes

[39]. Another difference between our experiments and those presented by Deutsch and Lee is the fact that they used PBL's which contained T cells, B cells and monocytes whereas we use purified T cells.

The role of two pore-domain channels in T cells has been doubted by some [1, 3] our results contribute to the emerging evidence contradicting this doubt, thus in summary we suggest that the KCNK5 up-regulation detected in CD3/CD28 activated human T cells helps ensure a hyperpolarization of the membrane thus favoring a sustained  $\text{Ca}^{2+}$  influx and T cell activation. We further suggest that to keep the cells from losing volume thereby supporting a strong proliferation the  $\text{Cl}^-$  channel VRAC is functionally down-regulated.

## Acknowledgements

The technical skills of Dorthe Nielsen (University of Copenhagen) and Pia Birn (Novo Nordisk) is gratefully acknowledged and appreciated. This work was supported by The Danish Council for Independent Research/Natural Sciences (grants 09-064182 and 10-085373) and The Lundbeck Foundation, Denmark (J Nr R32-A3102).

## Disclosure Statement

The authors declare that they have no conflict of interest.

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